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# ATP Binding to the $\sigma^{54}$-Dependent Activator XylR Triggers a Protein Multimerization Cycle Catalyzed by UAS DNA

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## Summary

**The events that take place at the prokaryotic enhancer of the *Pu* promoter of *Pseudomonas putida* prior to the engagement of the $\sigma^{54}$-RNA polymerase ($\sigma^{54}$-RNAP) have been studied in vitro. ATP hydrolysis by XylR, the cognate regulator of the system, is preceded by the multimerization of XylR at the enhancer, which is itself triggered by the sole allosteric effect of ATP binding to the protein. Since ADP is unable to support multimerization, ATP hydrolysis might be followed by a return to the nonmultimerized state. This notion is supported further by the properties of mutant proteins that seem to be frozen, in either the nonmultimerized or the multimerized state, respectively. These results support a cyclic mechanism of ATP-dependent association/dissociation of XylR at the promoter UAS that precedes any involvement of the polymerase in transcription initiation.**

## Introduction

Bacterial promoters dependent on the sigma factor $\sigma^{54}$ undergo a distinct mechanism of transcriptional activation that involves the binding of cognate regulatory proteins to upstream activating sequence (UAS) located >100 bp from the binding site of the RNA polymerase (RNAP). The activators that act in concert with $\sigma^{54}$ form a group of homologous proteins generically known as the NtrC family (Kustu et al., 1989, 1991), named after the most studied member of the group. With the only known exception of the LevR protein of Bacillus (Débarbouillé et al., 1991), the remaining components of the family display an ordered three-domain structure with different degrees of similarity among them (Morett and Segovia, 1993). The most divergent domain is the N-terminal module (the so-called A domain), a signal reception module that determines the activity of the protein (reviewed by Shingler, 1996). In two archetypes of the protein family, i.e., NtrC (Ninfa and Magasanik, 1986; Keener and Kustu, 1988; Klose et al., 1993) and DctD (Huala et al., 1992; Gu et al., 1994), the immediate signal received by the A domain is a phosphorylation effected, in each case, by a cognate kinase, as in bacterial two-component systems (Nixon et al., 1986). In other regulators, the A domain interacts directly with the chemical signal that they respond to, such as formate (the FhlA protein) (Hooper et al., 1994), toluene or xylenes (XylR) (Delgado and Ramos, 1994; see below), or phenol/cresols (DmpR) (Shingler and Moore, 1994; Shingler and Pavel, 1995). Finally, there are also cases in which the A domain plays only a small role in transcriptional activity, either because its control depends on an additional factor (such as the NifA protein, regulated by NifL) (Austin et al., 1990; Berger et al., 1994) or because the A domain does not exist as such (as is the case with HrpS) (Xiao et al., 1994). The signal reception module A is typically connected to the central portion of these proteins through a flexible hinge domain called Q linker (Wooton and Drumond, 1989). The central domains of all $\sigma^{54}$-dependent proteins are highly conserved, since they interact with the $\sigma^{54}$-RNAP holoenzyme (Berger et al., 1994; Lee and Hoover, 1995) and bear the ATPase activity (Weiss et al., 1991; Austin and Dixon, 1992; Lee et al., 1993, 1994; Austin and Lambert, 1994; Berger et al., 1994; Hopper and Böck, 1995) and the oligomerization determinants (Porter et al., 1993; Flashner et al., 1995) that are required for transcription initiation. Next to the central modules, the C-terminal domains are connected to the rest of the protein by a linker of variable length, and they include a helix-turn-helix motif responsible for the binding to DNA (North et al., 1993).

In spite of responding to very different stimuli, the $\sigma^{54}$-dependent activators seem to share a common mechanism of transcriptional activation (Kustu et al., 1991). This family of proteins typically binds to distant upstream target DNA sites (Reitzer and Magasanik, 1986). These must loop out, sometimes with the assistance of auxiliary proteins such as integration host factor (IHF) (Hoover et al., 1990; Gober and Shapiro, 1990; de Lorenzo et al., 1991; Claverie-Martin and Magasanik, 1991; Pérez-Martín et al., 1994) or HU (Pérez-Martín and de Lorenzo, 1995a) to contact the $\sigma^{54}$-RNAP already bound to the promoter (Su et al., 1990). Productive interactions between the activator and the enzyme result in isomerization of the closed complex into an open complex (Sasse-Dwigth and Gralla, 1988; Popham et al., 1989). This step is coupled to ATP hydrolysis mediated by the activator (Wedel and Kustu, 1995). Some findings on NtrC indicate that, at least in this case, the activator needs to multimerize in order to form a complex competent for ATPase activity (Porter et al., 1993). Such multimerization is assisted by the DNA of the cognate NtrC binding sites (Mettke et al., 1995).

Although the connection of ATP hydrolysis by this type of activator to transcription initiation has been well documented in recent years (mostly in the case of NtrC), not much is known on how the reception of the activating signal is transduced from the A domain to the rest of the protein. In fact, it seems that each protein follows a different pathway during its earlier stages, although they all are channeled later into a common activation mechanism. For instance, XylR, the activator of an operon of Pseudomonas putida for biodegradation of *m*-xylene (Marqués and Ramos, 1993; see below) bears an A domain that acts as an intramolecular repressor (Fernández et al., 1995). In this case, the A module of

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the protein down-regulates the otherwise constitutive activity of XylR through specific interactions with the central domain (Pérez-Martín and de Lorenzo, 1995b). Since XylR variants entirely deleted of the A domain are fully constitutive in vivo and in vitro (Fernández et al., 1995; Pérez-Martín and de Lorenzo, 1996a), it appears that the first step for activation of XylR upon binding of the aromatic effectors (i.e., *m*-xylene) to the A domain (Delgado and Ramos, 1994) is the release of such repression (Fernández et al., 1995). But what exactly follows the release of repression, and how is that connected to the hydrolysis of ATP that is itself coupled to transcription initiation? XylR is a particularly suitable protein through which to study this issue, since its specific activation steps (interaction with *m*-xylene and release of intramolecular repression) and the steps shared with other $\sigma^{54}$-activators (ATP hydrolysis and interaction with $\sigma^{54}$-RNAP) can be easily separated by using an XylR derivate deleted of its A domain. The results reported in this paper reveal a number of events that follow activation of XylR by *m*-xylene and are likely to be shared by the other members of the protein family. As shown below, the key step in the process is a major conformational change undergone by the protein upon ATP binding, which precedes and is independent of the hydrolysis of the nucleotide. This is followed then by an ATP-driven cycle of multimerization and demultimerization of active XylR that could occur at every round of transcription initiation.

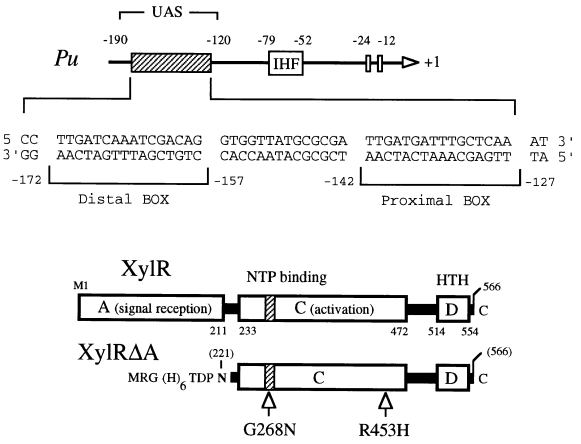


Figure 1. Organization of the XylR-Responsive Promoter and Its Enhancer Element

The distribution of relevant DNA sequences within the *Pu* promoter of the TOL plasmid is shown. These include UASs for XylR and the −12/−24 motif recognized by $\sigma^{54}$-RNAP. The promoter also contains a functional IHF binding site located within the intervening region. The boundaries of each of the XylR boxes (proximal and distal) at the *Pu* enhancer are indicated. The lower part of the figure shows the functional domains of XylR and its truncated and constitutive derivative XylRΔA. Relevant portions of the protein sequence include the signal reception N-terminal A domain, the central (C) module involved in NTP binding, and the D domain at the C-terminus, with a helix-turn-helix motif for DNA binding. The position of amino acid changes in the mutant proteins G268N and R453H described in the text are indicated with arrows. The leading residues of the XylRΔA protein, deleted entirely of the A domain but with a 6× His coil added at its N-terminus, is also indicated.

## Results

### ATP Binding to XylRΔA, Not Its Hydrolysis, Causes Cooperative Occupation of Its Binding Sites at the *Pu* Enhancer

During the course of previous studies on interactions of XylR with its target DNA sequences at *Pu* (Figure 1), we detected a difference between the nature of the interaction in vivo and in vitro. On the one hand, XylR binding in vitro to the two sites present at the *Pu* enhancer (termed, respectively, distal and proximal) (see Figure 1) is not cooperative, i.e., full occupation of the proximal site occurs at lower protein concentrations than does that of the distal one (Pérez-Martín and de Lorenzo, 1996a). On the other hand, we also observed that offsetting the two binding sites considerably decreased promoter activity in vivo, thus suggesting that protein–protein interactions between XylR dimers bound to each site were required for transcriptional activity (Pérez-Martín and de Lorenzo, 1996b). Since XylR has an intrinsic ATPase activity (Pérez-Martín and de Lorenzo, 1996a), we wondered whether these differences could be explained by the absence of ATP in the in vitro assay. To check this possibility, we reexamined the pattern of protection caused by the purified activator on the *Pu* enhancer in the presence of ATP (Figure 2A). It should be noted that in this and all subsequent experiments we employed a form of XylR that has been deleted of its N-terminus (XylRΔA) (see Figure 1) and is, therefore, locked in the form that follows release of intramolecular repression caused by *m*-xylene binding (Pérez-Martín and de Lorenzo, 1995b). As shown in Figure 2A, addition of ATP to the DNase I footprinting assays caused XylRΔA to display a marked cooperativity in the binding to the two sites of the *Pu* enhancer. Visual inspection of the footprinting patterns of Figure 2A indicated that, in the absence of ATP, increasing XylRΔA concentrations resulted in an earlier occupation of the proximal site (evidenced by the DNase I-hypersensitive band at position −141), followed by an occupation of the distal site (indicated by the hypersensitive band at −171). The presence of ATP altered this pattern, so that both sites appeared to be simultaneously occupied at lower protein concentrations. In addition, some changes beyond the boundaries of the two sites could be observed such that DNA regions outside the enhancer appeared weakly protected as well (Figure 2A). These effects suggested that ATP altered the mode of interaction of XylRΔA with its target DNA sequences. Interestingly, neither cooperative occupation of the enhancer nor protection beyond the two binding sites could be noticed when the two target sequences were offset by one half-helix turn of DNA, even in the presence of ATP (data not shown).

Since XylRΔA protein has an instrinsic ATPase activity that is strongly stimulated by UAS DNA in vitro (Pérez-Martín and de Lorenzo, 1996a), we sought to determine whether cooperative and extensive occupation of the *Pu* enhancer by the activator required ATP hydrolysis. For this, we made additional DNase I footprinting experiments in the presence of ATPγS, a nonhydrolizable ATP analog, or ADP, as specified in Figure 2B. Furthermore, to narrow down the region under study, we generated

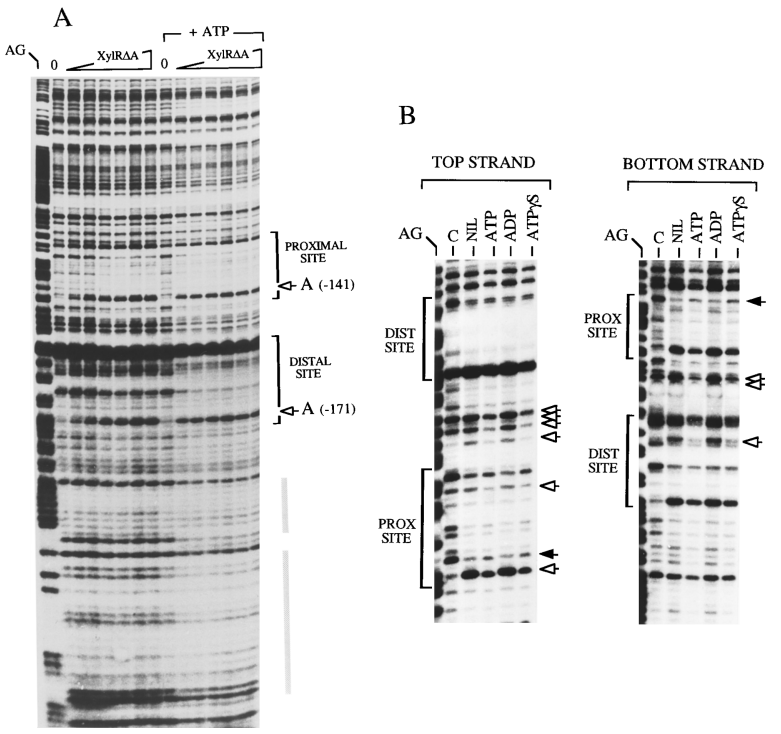


Figure 2. ATP Binding, Not Its Hydrolysis, Triggers Cooperative Occupation of the *Pu* Enhancer by XylRΔA

(A) DNase I footprints caused by increasing amounts of XylRΔA protein (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μM) bound to the *Pu* promoter in the presence or absence of 5 mM ATP. The DNA fragment used was a 0.5 kb EcoRI–PvuII segment containing the wild-type *Pu* promoter and labelled at its EcoRI end (closer to the enhancer). The XylR binding sites are indicated along with the DNase I hypersensitivity bands that are distinctive for each site. Note the intense protection of the distal site in the samples with ATP and the weak protection of the neighboring region below (indicated with bars). The AG is the result of the Maxam and Gilbert (1980) A+G reaction used as a reference.

(B) The footprints shown are the result of the binding of 0.5 μM XylRΔA to restriction fragments spanning exclusively the *Pu* enhancer, in the presence of 5 mM of each of the nucleotides indicated. C: Control: no XylRΔA; NIL: +XylRΔA, but without nucleotides. The organization of the EcoRI–PvuII restriction fragments used (see Experimental Procedures) permitted selective labeling of each strand of the DNA sequence of the enhancer shown in Figure 1. Some changes in the protection pattern caused by the addition of ATP or ATPγS (but not ADP) are noted to the left of the gels with empty arrows (protected bands) or solid arrows (overdigested bands).

DNase I footprints of both strands of a shorter DNA fragment (79 bp) exclusively spanning the *Pu* enhancer (Pérez-Martín and de Lorenzo, 1996b). The results shown in Figure 2B clearly indicated that ATP hydrolysis was not required for cooperative occupation of the UAS, since ATPγS gave rise to footprinting patterns identical to those caused by ATP. This is not true, however, for ADP, which fails to promote cooperative occupation of the sites. It seems, therefore, that ATP binding itself, and not its hydrolysis, is the cause of the effects observed. A closer inspection of Figure 2B revealed that, besides cooperative binding and extended protection of the enhancer by XylRΔA, the presence of ATP and ATPγS (but, again, not ADP) also elicited a number of subtle but perfectly reproducible changes within the two binding sites and the hinge sequence between them. These results suggested that ATP binding to XylR brings about interactions between proteins bound to separate sites that deform the intervening DNA region and also engage the adjacent nucleotide sequences.

### DNA and ATP Binding Drive the Multimerization of the XylRΔA Protein at the *Pu* Enhancer

A plausible hypothesis to explain the protection patterns of the *Pu* UAS by XylRΔA in the presence of ATP (Figure 2) was that binding of the nucleotide caused the multimerization of the activator through protein–protein interactions. This possibility was investigated through chemical cross-link assays with *bis*-maleimidohexane (BMH) (see Experimental Procedures), a homobifunctional reagent that reacts irreversibly with sulfhydryl groups. As shown in Figure 3, when ATP, ATPγS, or GTP (which is hydrolyzed by XylRΔA ten times slower than ATP) (data not shown) were present in the cross-linking reactions,

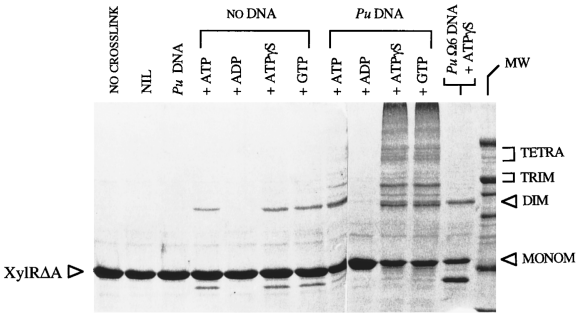


Figure 3. Chemical Crosslink Assays for ATP- and DNA-Mediated Multimerization of XylRΔA

The purified XylRΔA protein at 1 μM was incubated with the sulfhydryl cross-linking agent BMH in the presence of the nucleotides indicated and UAS-containing DNA (1 μM; supercoiled plasmid harboring the wild-type *Pu* promoter or a derivative, *Pu*Ω6, bearing the two XylR binding sites offset by the insertion of 6 bp). Samples were analyzed in an SDS–PAGE system as shown. The sample NIL was treated with BMH only. Maximum multimer formation occurs in the samples with UAS DNA and the nonhydrolyzable nucleotide ATPγS or the slowly hydrolyzed GTP. Faster migrating protein forms may correspond to internally cross-linked products (see text). The approximate location of the dimers, trimers, and tetramers is indicated to the right. Note that combinations of internally and externally cross-linked proteins give rise to a repertoire of multimeric forms with different mobilities in the gel.

higher molecular weight forms of the activator became apparent. Formation of such multimers was dramatically enhanced when DNA carrying the *Pu* enhancer was also added to the assays. The same DNA, but with the two XylRΔA binding sites offset by a half-helix turn, had little stimulatory effect. Similarly, addition of nonspecific DNA or a sequence bearing only one XylR binding site had no influence on multimer formation (data not shown). Consistent with the observations made on occupation of the UAS by XylRΔA (see above), ADP was unable to sustain formation of high molecular weight forms of the activator, even in the presence of *Pu* DNA. Taken together, the results shown in Figure 3 indicated that both ATP binding and specific DNA (i.e., a sequence bearing the two XylR binding sites in the same face of the DNA helix) are required for optimal multimerization of the activator at the *Pu* enhancer.

### ATP Binding Induces a Major Conformational Change in XylRΔA

Besides formation of XylRΔA multimers in the presence of ATP and UAS DNA, the cross-linking experiments of Figure 3 also revealed that the mere exposure of the activator to either ATP or its analogs ATPγS and GTP gave rise to a protein form that migrated in the gel below the band corresponding to the XylRΔA monomer. One possible explanation for this band was that, by acting intramolecularly, the cross-linking agent could fix a more compact form of the protein resulting from a conformational change induced by ATP binding. To investigate this possibility, we subjected the purified protein, under various conditions, to limited proteolysis with chymotrypsin. These assays are instrumental in revealing gross conformational changes caused by ligand binding (Schreiber et al., 1988; Tan and Richmond, 1990; Keidel et al., 1994). For the experiments shown in Figure 4, the activator was diluted to a concentration (0.1 μM) that did not support formation of multimers in the absence of DNA (see above) and was incubated with the different nucleotides indicated in each case. Increasing concentrations of chymotrypsin were then added to the samples, which were briefly incubated and analyzed in an SDS–polyacrylamide gel electrophoresis (SDS–PAGE) system, as shown in Figure 4. The profile of digestion products resulting from proteolysis of the activator depended on the nucleotide added. ATP and ATPγS caused XylR to display a quite different cutting pattern than XylRΔA by itself. However, the patterns obtained with ATP and ATPγS were identical. On the contrary, ADP had no effect on the cleavage pattern of the activator with respect to XylRΔA in the absence of additions. The effect of ATP and ATPγS could not be attributed to an influence of the nucleotides in the activity of the protease, as the digestion pattern of bovine serum albumin was unchanged regardless of the nucleotide added (data not shown). Additional assays were carried out using other proteases, i.e., subtilisin, V8 protease, and trypsin, with similar results (data not shown). Furthermore, the changes detected are not the result of multimer formation, since, as mentioned above, the assays were made under conditions in which addition of the cross-linking reagent BMH did not yield any high

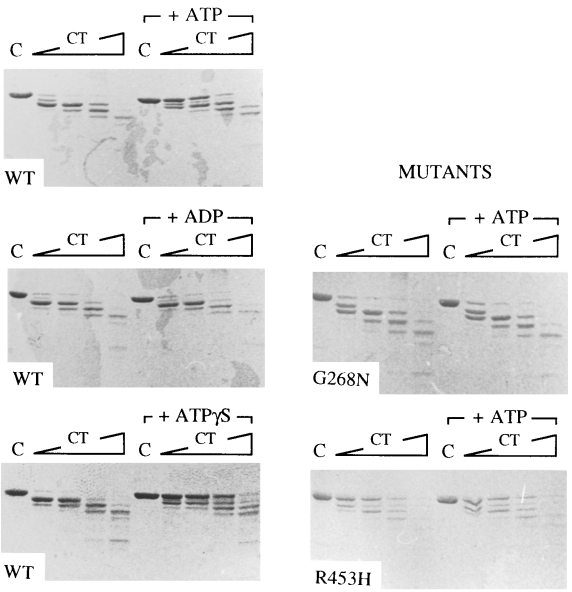


Figure 4. Limited Proteolysis of XylRΔA and Mutant Derivatives G268N and R453H, in the Presence of Different Nucleotides

Native XylRΔA protein or its mutant variants G268N and R453H were diluted to 0.1 μM, briefly preincubated with 5 mM of the nucleotides indicated, and subjected to partial proteolysis with increasing concentrations of chymotrypsin (1.0, 2.5, 5.0, and 10 μg/ml) for 15 min at room temperature. After TCA precipitation, the digestion products were visualized in a 10% SDS–PAGE. Note the change in the cleavage pattern of XylRΔA with ATP and ATPγS, but not with ADP. Note also that the pattern presented by the mutants is not altered by addition of ATP, although that of G268N is similar to XylRΔA with no additions and that of R453H resembles the nonmutant protein with ATP/ATPγS (see text for explanation).

molecular weight products (data not shown). Therefore, the conclusion of these experiments is that ATP (or ATPγS) binding to XylRΔA caused a significant conformational change in the activator and, furthermore, that ATP is by itself an allosteric effector of the protein, regardless of its subsequent hydrolysis.

### Interaction between DNA-Bound XylRΔA Proteins Induces ATPase Activity

We have reported previously that, like other members of the NtrC family (Porter et al., 1995; Mettke et al., 1995), XylRΔA has an ATPase activity that is strongly dependent on protein concentration and DNA bearing a wild-type *Pu* enhancer (Pérez-Martín and de Lorenzo, 1996a). In view of the results reported above on DNA-dependent association of XylRΔA, it seems most likely that the ATPase activity becomes apparent only in the multimerized form of the protein. Should this be true, the ATPase activity is expected to be sensitive to the arrangement of the XylR binding sites within the enhancer. To verify this prediction, we examined the ATPase activity of XylRΔA in the presence of mutant derivates of the *Pu* enhancer that bear insertions of different lengths between the two XylR binding sites (Pérez-Martín and de Lorenzo, 1996b). These insertions rotate each binding site by either one half-turn or an integral turn with respect to the other, so that, regardless of the distance, the binding sites are either on the same

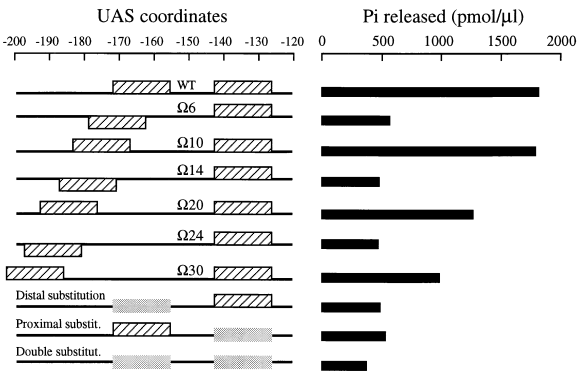


Figure 5. Dependence of the ATPase Activity of XylRΔA on the Phasing of the Binding Sites at the *Pu* Enhancer

Enzymatic assays were carried out, as indicated in Experimental Procedures, by mixing 1 μM of purified XylRΔA along with 2 μM of each of a series of equivalent supercoiled plasmids bearing *Pu* promoter derivatives in which the two XylR binding sites were separated by the number of base pairs indicated, or substituted with a heterologous sequence. The figure notes the offsetting or resetting of the binding sites in each case.

side of the DNA helix or on opposite faces. The results summarized in Figure 5 clearly indicated that ATPase activity decreased significantly when the XylRΔA binding sites were offset. Not surprisingly, the ATPase activity was low when the DNA added to the assays carried only one XylR binding site. To further determine the nature of the increased ATPase activity under conditions that promote multimerization, we examined the $V_{max}$ and $K_m$ of the reaction under different conditions. Measurements of the rate of ATP hydrolysis indicated that the effect of adding *Pu* enhancer DNA to the reaction (i.e., the stimulation of multimerization) was that of increasing the apparent enzymatic activity (apparent $V_{max}$ at 1 μM of activator changed from 0.4 to 4.0 nmol/min), while affinity of XylRΔA for ATP did not change significantly (apparent $K_m$ at 1 μM varied only from 1.5 mM to 2.3 mM in the presence of DNA). These observations suggest that ATP binding and subsequent multimerization of XylRΔA at the UAS are a prerequisite for ATPase activity. The results presented so far support the notion that there are a number of intermediate steps between the activation of XylR by *m*-xylene and the hydrolysis of ATP that is connected to transcription initiation. Since ADP is unable to sustain the assembly of the multimeric form of XylRΔA with ATPase activity, it is possible that the upstream nucleoprotein complex breaks apart after ATP is hydrolyzed. This hypothesis is supported further by the properties of two XylRΔA mutants discussed in the following sections.

### XylRΔA G268N Mutant Is Defective in Both Binding ATP and Multimer Formation

From the data above, it seems that the key event that follows release of intramolecular repression of XylR by *m*-xylene (Pérez-Martín and de Lorenzo, 1995b) is the conformational change caused by ATP binding to the protein. As a consequence, mutants that cannot bind ATP are predicted to be incapable of occupying cooperatively the *Pu* enhancer or forming ATP-dependent multimers. To test directly the pivotal role of ATP in this process, we have examined the properties of a mutant activator incapable of ATP binding because of a point mutation in the Walker A domain of the sequence (North et al., 1993) involved in nucleotide binding (Pérez-Martín and de Lorenzo, 1996a). The XylRΔA derivative G268N (see Figure 1) does not significantly bind ATP, compared with the unchanged XylRΔA; as a consequence, it has no ATPase activity, even at high protein concentrations, and is unable to activate transcription (Pérez-Martín and de Lorenzo, 1996a). However, since the change present in the mutant exclusively affects the nucleoside triphosphate (NTP) binding pocket of the activator, the rest of the protein remains unaltered. This mutant was examined, therefore, under the assumption that it would represent the form of the protein that is frozen in the stage prior to ATP binding. The purified mutant protein was passed through the same battery of tests that were previously employed on native XylRΔA, namely, DNase I footprinting, multimerization assays, and limited digestion with chymotrypsin. The results of these assays, shown in Figure 4 and Figure 6, indicate that, under all conditions tested, G268N behaved identically to the native XylRΔA protein in the absence of ATP. For instance, the DNase I footprints shown in Figure 6A indicated that, regardless of the addition of ATP, G268N protein binds its target DNA sequences within *Pu* with the same apparent affinity and with the same protection pattern as the native XylRΔA without ATP. Similarly, the cross-linking experiments of Figure 6B show that G268N was completely unable to form multimers even under the best conditions applied to XylRΔA. Finally, limited proteolysis of G268N resulted in a cleavage pattern that was not altered by the presence of ATP assays and is indistinguishable from the native XylRΔA protein in the absence of the nucleotide (see Figure 4). These results confirm that the changes effected by ATP to XylRΔA are directly related to the binding of the nucleotide to the protein and not to any other indirect effects.

### XylRΔA R453H Mutant Is Fixed in a Multimeric State, but It Is Not Functional

During the course of a previous study (Pérez-Martín and de Lorenzo, 1996a), we generated an XylRΔA variety named R453H. The amino acid change in this mutant protein was located within a portion of the central domain of XylR (see Figure 1) that is highly conserved among the members of the NtrC family of activators (Morett and Segovia, 1993). Since R453H is able to bind ATP but does not have ATPase activity, we reasoned (Pérez-Martín and de Lorenzo, 1996a) that the mutation could bear a second site involved in enzymatic activity. In view of the results presented above, it became plausible that R453H could be affected in the multimerization that is required for ATP hydrolysis. The behavior of the purified mutant protein in footprinting experiments, multimerization assays, and limited proteolysis (see Figure 4; Figure 6) revealed that that was apparently the case, although, unexpectedly, it behaved as if locked in a multimeric state. This conclusion is based on the following observations. The DNase I footprint of Figure 6B showed that, regardless of ATP addition, the binding

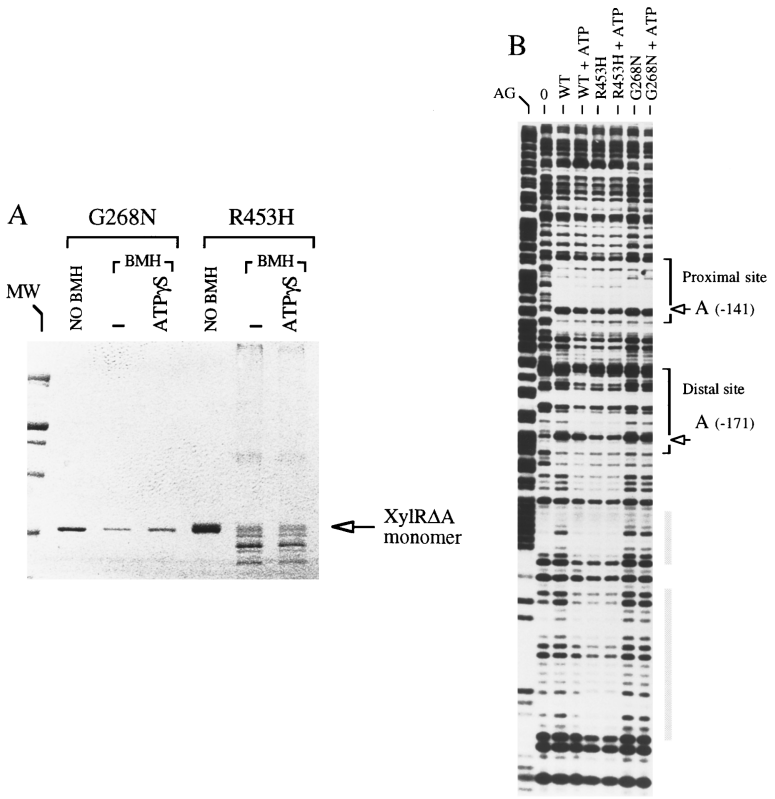


Figure 6. Properties of XylRΔA Mutant Derivatives G268N and R453H

(A) Multimer formation. The two purified proteins were subjected to the same cross-linking assay with BMH described in the legend to Figure 3, with UAS-containing supercoiled plasmid added to all samples but ATPγS added only to those indicated on top of the gel. Note that G268N seems to be frozen in a nonmultimerized form, while R453H presents multimerized and internally cross-linked products regardless of nucleotide addition.

(B) Interactions with DNA. The footprint shown is the result of the binding of 0.5 μM XylRΔA, G268N, or R435H proteins, with or without ATP, to the same restriction fragment used in the experiment shown in Figure 2A. Consistent with the multimer formation assays, G268N gives rise, regardless of ATP addition, to a protection pattern identical to that of wild-type XylRΔA without ATP. On the contrary, R453H produces in all circumstances a profile of bands similar to that caused by wild-type XylRΔA with ATP.

of R453H to target DNA sequences at the UAS of the *Pu* promoter resembled that of native XylRΔA in the presence of ATP. In addition, chemical cross-linking of the protein resulted in multimers and internally cross-linked products independently of ATP addition (Figure 6A). However, unlike the case of the wild-type XylRΔA protein with ATPγS (see Figure 3), multimers of defined intermediate size were poorly detected. This was interpreted as an indication of the very stable effector-independent association of the mutant as well as of an enrichment in internally cross-linked products fixed in conformations unable to interact between them. In addition to the footprints and the cross-linking assays, the patterns of limited proteolysis of R453H with chymotrypsin (see Figure 4) were not affected by ATP and resembled those obtained with the native XylRΔA protein in the presence of ATP or ATPγS. These results suggested that the R453H mutant is frozen in an oligomeric, but nonfunctional, state that is independent of ATP binding. It is therefore possible that the behavior of this mutant reflects a need for the disassembly of the XylRΔA multimer at the UAS of the promoter for continued ATPase activity and transcription initiation.

## Discussion

Activation of $\sigma^{54}$-dependent promoters require ATP hydrolysis by the regulator (Popham et al., 1989; Weiss et al., 1991; Lee et al., 1993, 1994; Pérez-Martín and de Lorenzo, 1996a). This has been demonstrated extensively for the archetypical regulator NtrC, where ATP hydrolysis is coupled energetically to open complex formation by the RNA polymerase (Wedel and Kustu, 1995), perhaps through activation of the sigma factor itself (Wang et al., 1995). In this work, we show that, at least in the case of XylR but most likely in all $\sigma^{54}$-dependent regulators as well, ATP is involved not only in activation of the polymerase to initiate transcription but also in a number of earlier molecular events that occur at the UAS, prior to any engagement of the enzyme in the process. In our view, the most relevant piece of information revealed by the experiments shown in this article is that ATP is both the initiator and the driving force of a cycle of protein multimerization and demultimerization of XylR at the UAS of the *Pu* promoter once XylR is released from its intramolecular repression. ATP is therefore an allosteric effector of XylR that triggers the process that results in its eventual hydrolysis and transcription initiation. As shown above, ATP binding to activated XylR causes, by itself, a major conformational change that makes the activator competent to assemble in a multimeric form at the UAS of the promoter. Only then does the ATPase activity of the protein become apparent.

The data shown in this work shed additional light on the role of ATP in $\sigma^{54}$ promoters (Wedel and Kustu, 1995). As shown in the scheme presented in Figure 7, the entry of ATP takes place during an early stage of the process, so that the nucleotide binds to an already preactivated XylR molecule resulting from either *m*-xylene binding or removal of its A domain. ATP then induces a conformational change in XylR that stimulates protein–protein interactions between activator molecules bound to the

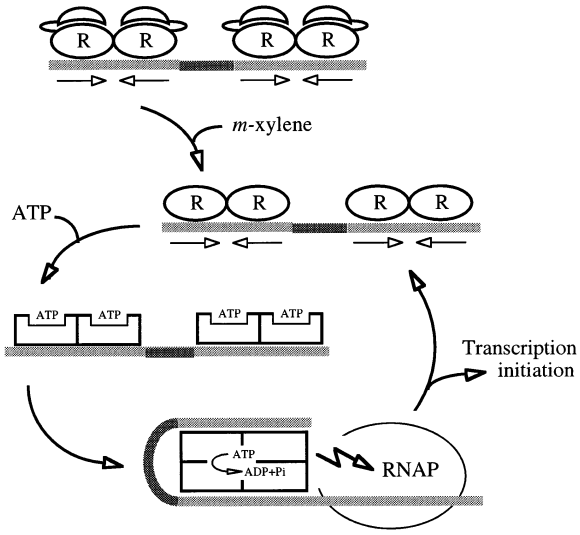


Figure 7. A Model for the Multimerization Cycle of XylR at the UAS of the *Pu* Promoter

The series of events that follow the release of intramolecular repression caused by *m*-xylene binding to the A domain of XylR are sketched in the figure as a multimerization and demultimerization cycle driven by ATP. The XylR protein bound to its cognate sequences at the *Pu* enhancer undergoes a conformational change upon ATP binding, which makes the protein competent to form a multimer. This may involve just a spatial rearrangement of the proteins already bound (as represented in the figure) or, alternatively, it could also engage additional XylR molecules that are recruited to the proximity of the enhancer. The multimer is then able to hydrolyze ATP and channel the released energy into transcription initiation by the polymerase. Since ADP is unable to sustain the multimer, it is likely that the complex returns to a nonmultimerized state after ATP hydrolysis.

UAS. Multimer formation is catalyzed by the DNA of the region that bears two phased binding sites. Since the stoichiometry of the multimer is unknown, we cannot distinguish at this point whether only XylR dimers prebound to the UAS are involved in the interactions or whether more XylR units are recruited from solution to associate to the complex. Since XylR does not seem to be an abundant protein in vivo (data not shown), it is likely that the functional multimer does not go beyond a limited number of units, although this point requires further investigation. In any case, once the multimer is formed, the ATPase activity is switched on and ATP hydrolysis could be coupled to activation of the polymerase, like other $\sigma^{54}$-dependent proteins, i.e., NtrC (Wedel and Kustu, 1995). Our results suggest that hydrolysis of the nucleotide and production of ADP contribute to multimer disassembly and a return to pre-ATP binding conditions. This notion is based on two independent observations. First, the results of Figure 3 show that the yield of multimeric forms increases greatly when XylRΔA/DNA mixtures were added with slowly hydrolyzed GTP or ATPγS. Since cross-linking is a slow reaction in itself (see Experimental Procedures), this occurrence is interpreted as the result of a longer-lived multimer when the effector cannot be hydrolyzed. This situation should differ from the progressive dissassembly of the complex as long as ATP becomes cleaved by XylRΔA. The concept of a cycle is supported, in addition, by the properties of the two mutants G268N and R453H. In the first case, the cycle seems to be stopped at the very early stage (ATP binding), so that G268N protein appears to be frozen in the nonmultimerized state. Interestingly, a second mutant, R453H, behaves as if frozen in the multimeric state, yet it is unable to hydrolyze ATP and cannot activate transcription. It therefore looks like the process functions in a cycle of association and dissociation every time ATP is hydrolyzed.

The archetypical regulator NtrC has been defined as a simple molecular machine (Porter et al., 1993; Wedel and Kustu, 1995), a concept that can surely be applied to all $\sigma^{54}$-dependent regulators. The results on XylR presented in this work add a wider dimension to the concept, since they permit us to envision such a machine in the context of a cycle of different conformations and multimeric states driven by the binding and hydrolysis of ATP that is reminiscent of those found in various transducing systems (Webb, 1992; Bourne et al., 1991). The function of the cycle might be to impart directionality and order to contacts between multiple components, thus increasing the range and fidelity of macromolecular interactions (Alberts and Miake-Lye, 1992). This raises some questions on the precise mechanism by which ATP hydrolysis causes formation of the open complex in $\sigma^{54}$ promoters. In fact, such hydrolysis is not strictly required, since $\sigma^{54}$ mutants are available that initiate transcription without any regulator (Wang et al., 1995). This suggests that the series of complex events that precedes the engagement of the polymerase in the process may have, as the final and unique target, the activation of the sigma factor itself.

## Experimental Procedures

### DNA Footprinting

The DNase I footprinting procedures and the purification of XylRΔA, G268N, and R453H are described in detail in Pérez-Martín and de Lorenzo (1996a). The DNA segment used for the experiment of Figure 1A, bearing the entire wild-type *Pu* promoter sequence, was obtained from plasmid pEZ9 (de Lorenzo et al., 1991) as an EcoRI–PvuII fragment. Where indicated, the footprinting mixtures were added with ATP or other nucleotides at 5 mM. The segments used for the experiment of Figure 1B bear a 79 bp XbaI insert spanning the entire *Pu* enhancer and were obtained as EcoRI–PvuII fragments from plasmids pUC/UA (for labeling of the bottom strand) (Figure 1) and pUC/UB (for labeling of the top strand), as described in Pérez-Martín and de Lorenzo (1996a).

### Protein Cross-Linking Assays

In vitro formation of protein multimers was examined in 100 μl reactions containing 2 μM of purified XylRΔA protein or its mutant derivates G268N and R453H (Pérez-Martín and de Lorenzo, 1996a) in 20 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 40 mM KCl, and the additions of nucleotides (5 mM, final) or DNA (1 μM, final) indicated in each case. In the samples where DNA was added, it was in the form of a supercoiled plasmid containing either the wild-type *Pu* promoter sequence (pUC*Pu*) or an equivalent construction (pUC*Pu*Ω6) in which a 6 bp insert had been placed between the two XylR binding sites so that they are offset by half-helix turns (Pérez-Martín and de Lorenzo, 1996b). Reactions were prepared on ice and then preincubated at 30°C for 5 min, after which they were added with 10 μl of a fresh 1/100 dilution in water of a 10 mg/ml stock solution in dimethyl sulfoxide of the cross-linking reagent BMH (Pierce). The maleimide groups react specifically and irreversibly with sulfhydryl groups under mild conditions, and free −SH

groups may thereby become cross-linked either intermolecularly or intramolecularly. The reaction setup leaves BMH at a final concentration of 10 μg/ml. Control samples without BMH contained 0.1% dimethyl sulfoxide. Reactions were allowed to proceed for 30 min at room temperature, after which they were stopped by addition of 10 μl of 1.4 M β-mercaptoethanol and 11 μl of 100% tricarboxylic acid (TCA). After 1 hr at 4°C, the precipitated proteins were collected, washed twice with 70% cold ethanol, and resuspended in 20 μl of 1× protein sample buffer with 2% SDS and 5% β-mercaptoethanol. Samples then were heated briefly at 95°C prior to separation in an 8% polyacrylamide SDS–PAGE system, followed by staining with Coomassie brilliant blue.

### Limited Proteolysis Experiments

Partial proteolysis assays to detect gross conformational changes (Schreiber et al., 1988; Tan and Richmond, 1990; Keidel et al., 1994) were carried out in sample volumes of 100 μl containing in all cases 0.1 μM of purified XylRΔA protein or its mutant derivates G268N and R453H (Pérez-Martín and de Lorenzo, 1996a). The reaction buffer was identical to that used for the chemical cross-linking assays described above. The mixtures of the proteins and the nucleotides (at 5 mM) indicated in each case were prepared on ice and preincubated for 5 min at room temperature. The proteolysis reactions were then started by the addition of 2 μl of adequate dilution of chymotrypsin (Boehringer) in the same buffer, so that the final concentrations in the assay samples were 0, 1.0, 2.5, 5.0, and 10 μg/ml. Reactions were incubated for 15 min at room temperature and then stopped by the addition of 10 μl of 100% TCA. The digested proteins were then processed and analyzed as described above.

### ATPase Assays

Hydrolysis of ATP was measured through the release of radioactive orthophosphate ($^{32}$Pi) from [$\gamma$-$^{32}$P]ATP as described in detail elsewhere (Pérez-Martín and de Lorenzo, 1996a). Where indicated, supercoiled DNA (1 μM) was added to the reactions. This included pUC*Pu* (bearing the promoter sequence with the wild-type UAS) (see above), as well as equivalent plasmids containing promoter variants with helical insertions in their upstream region, which increased the distance between the XylR binding sites by 6, 10, 14, 20, 24, or 30 bp, thus offsetting and resetting, alternatively, the relative positioning of sites on the DNA helix. Other plasmids employed in the experiments contained UAS regions in which each of the XylR binding sites of the *Pu* enhancer had been replaced by a heterologous sequence (Pérez-Martín and de Lorenzo, 1996b).

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